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14. ABSTRACT The binding of TGF- β family molecules to their receptors on the surfaces of cells initiates a signaling pathway within the cells. The Smad family of molecules mediates the propagation of the intracellular signal from the receptor for TGF- β . Two different Smad molecules are essential to propagating this signal, Smad 2 and Smad 3. It has been shown that Smad 2 expression is altered in prostate cancer and adult normal prostate cells enter programmed cell death with increased Smad 2 expression. In the analysis of prostate development the expression of Smad 2 is increased at the very early stages of gland development in the first 7 days post-natally. The pattern of Smad 2 expression is indicative of TGF- β signaling and during early gland formation with cell proliferation. Smad 3 is also expressed in the early stages of gland development and continues to be expressed at late stages of development. Phosphorylation of the Smad molecules is essential to signal propagation and both Smad 2 & 3 are phosphorylated indicating that TGF- β signaling is occurring. Prostate development demonstrates the involvement of the TGF- β signaling pathway in mechanisms that stimulate cell division, a process required for tumorigenesis. Differential response of prostate cells based on age of the tissue may indicate changes in response to the growth factors and persistence or reacquisition of the neonatal phenotype may be associated with tumor development. These results on the basic understanding of the signaling pathway will lead to new strategies for therapeutic intervention to alter cell growth and differentiation that may lead to neoplastic events.					
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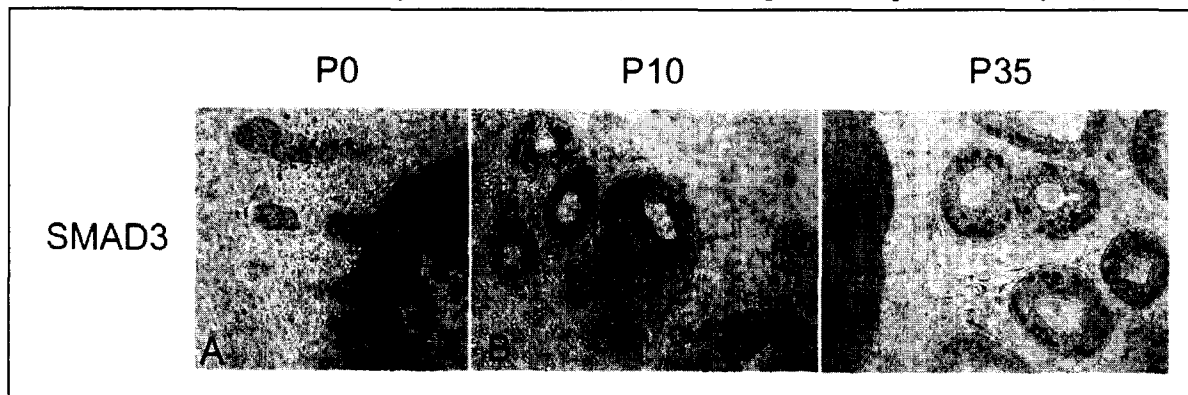
Introduction:

The overall theme for this study focuses on the role of TGF- β signaling in normal prostate development. TGF- β growth factors bind to a heteromeric surface receptor (T β IR/T β IIR). This binding results in a cascade of intracellular signaling mediated by the Smad family of proteins with Smad2,3 and 4 up-regulating the signaling and Smad6,7 inhibiting the signaling. The mechanisms of signal transduction by growth factors represents an important area of investigation to understand fetal development and tissue differentiation. Resumption of patterns of gene expression that are typically embryonic has been associated with tumorigenic conversion of cells. Our studies are focused on the transduction of signal by transforming growth factor- β (TGF- β) during development. Smad 2 and Smad 3 are activated following ligand binding to specific receptors for TGF- β and the molecules must be phosphorylated to be active in changing gene transcription. The on-going studies are characterizing patterns of Smad gene expression during prostate gland development to examine the effects on cell growth and differentiation. The studies are focused on the molecular mechanism of TGF- β signal transduction and the role of these mechanisms in morphogenesis.

Body:

Specific Aim 1. To define the developmental and temporo-spatial expression of Smad genes during embryonic prostate development (i) in vivo and (ii) in neonatal mouse prostate organ culture.

The expression of Smad 2 and Smad 3 has been characterized in the developing prostate gland. Previously we have shown that Smad 2 expression and post-translational activation by phosphorylation is critical for the intracellular propagation of the TGF- β signal. The earliest periods of organ development are marked by a dramatic increase in Smad 2. This Smad 2 is phosphorylated indicating that it has responded to the binding of the growth factor ligand to the signaling receptor. Our most recent results have shown that Smad 3 is also expressed in the proliferating prostate tissue at critical stages of organogenesis (Figure 1). The expression of Smad 3 demonstrates several important features; 1) Smad 3 is present at postnatal day 0 (P0) in a



distribution that is the same as Smad 2 indicating that this molecule is active in the intracellular signaling pathway. As can be seen Smad 3 continues to be expressed at postnatal day 35 (P35) a point when Smad 2 is no longer observed. This result indicates that Smad3 is probably activated by other members of the TGF- β signaling family providing evidence that the cell surface binding of growth factors progresses through different stages during development and ultimately tissue maturation. These findings are consistent with the results reported by others in the literature.^{1,2} We are continuing to investigate these findings using a genetically engineered mouse that drives

Smad2 under the control of an epithelial-specific promoter, which should result in the forced expression of Smad 2 in the more mature tissue and consequently provide information relative to the effects on the tissue when Smad2 is present in excess of normal.

Status of Specific Aim 1.

1a: Original reagents and protocols developed and in use, new approaches continuing to be developed to allow the optimal analysis of the mechanisms - completed.

1b: Initial characterization of baseline descriptive distribution of mRNA is completed and these studies continue as the basis for establishing the controls in each experiment to determine the impact of specific interventions - completed.

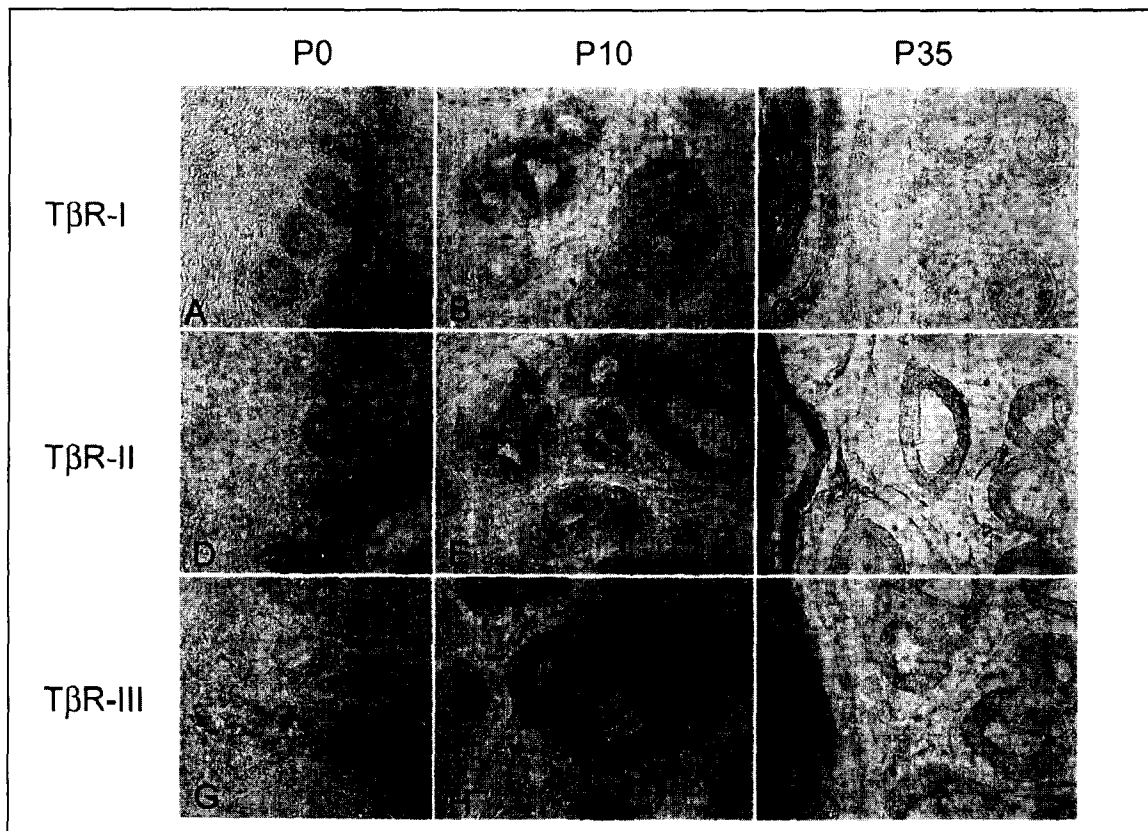
1c: Initial characterization of the baseline descriptive distribution of protein is completed and these analyses continue as outcome measures for control and experimental groups - completed.

Publication:

XM Cui, N Shiomi, CF Shuler Smad 2 and Smad 3 expression critical to prostate organogenesis, (to be submitted) Anatomy and Embryology,

Specific Aim 2. To determine the molecular mechanisms of TGF- β pathway restricted Smad2 and Smad3 in regulating prostate ductal branching morphogenesis and cytodifferentiation in serumless organ culture.

A critical feature in evaluating the mechanisms of Smad2 and Smad3 mediated signaling is the relationship to the specific TGF- β receptors on the surface of the cells. In this respect specific findings have shown a distinct temporal relationship between the expression and distribution of the 3 TGF- β receptors and the development of the organ (Figure 2). The earliest



developing tissues exhibit all three of the TGF- β receptors in the proliferating tissues (P0). At 10 days after birth P10 the receptors are more localized to specific cells in the developing organ demonstrating the TGF- β signaling and Smad 2/3 mediation the signal are localized in specific cells. The P10 results also demonstrate that the T β R-III receptors becomes a major receptor at that age demonstrating changes in the pattern of TGF- β signaling and Smad 2/3 mediation of the signal. The correlation of the receptor results and the distribution of the Smad 2/3 expression as observed in Specific Aim 1 demonstrates an important finding that Smad 3 remains expressed at P35 a point when none of the 3 TGF- β receptors are found in the prostate tissue. The important role for this growth factor family in prostate development has been shown by others.^{2,3,4} This provides further evidence that the mechanisms regulating Smad 2 and Smad 3 may be related to different growth factor ligands and that multiple Smad-mediated mechanisms may be active in the tissues during development. These results will continue to be expanded using the genetically engineered mice over-expressing Smad 2 in the later stages of organ development.

Our studies continue to examine the effects of siRNA on the differentiation of the organ and the expression of specific Smad genes and TGF- β pathway molecules.. The siRNA approach is a new strategy that effectively knocks-down gene expression without the associated toxicity. The Dharmacon Company has developed siRNA reagents with appropriate controls for all the molecules in the TGF- β signaling pathway allowing the experimentation to be carefully planned and controlled. Further studies are on-going to use the siRNA approach to alter the TGF- β signaling and determine the impacts on cell proliferation and cell death.

The effects of excessive expression of Smad 2 have been analyzed in a genetic model developed to address some deficiencies of the originally proposed adenovirus approach. Originally it was proposed to use an adenovirus model to transfer new genetic elements into the cell however it was found that there was sufficient delay in the expression of the DNA and presence of the new protein that critical developmental events were completed before the new gene had an effect. A transgenic animal model has been developed that permits overexpression of Smad 2 in epithelial cells. An epithelial cell specific promoter, K14, has been linked to the Smad 2 gene and transgenic mice generated. This DNA promoter-gene construct has been shown to greatly increase Smad-2 expression in cells in transgenic animals. We are utilizing this model system to advantage to analyze the effects of Smad-2 expression on the development of the prostate. If Smad 2 is directly linked to cell proliferation then increased levels of Smad 2 should be linked to enlargement of the organ and replicate events that are observed in benign prostatic hyperplasia and in prostatic carcinoma. This transgenic mouse model is a unique approach to examine gain-of-function outcomes on the developing tissue

Another strategy that is being developed to analyze tissue-specific expression patterns linked to TGF- β signaling uses a TGF- β promoter cloned into a DNA construct that contains the sequences for two genes, GFP (green fluorescent protein) and Cre (Cre recombinase). This animal model will permit the patterns of expression of TGF- β to be analyzed in specific tissues through the GFP and allow tissues specific elimination of genes by Cre. The DNA constructs have been produced and injected into fertilized oocytes. These oocytes have been used to generate lines of mice with this unique pattern of gene expression. Breeding of the mice is continuing to generate sufficient numbers of animals to determine if the transgene expression goals have been met. Once this has been ascertained these animals will result in a unique model to specifically characterize the role of defined genes during organogenesis. We have secured through a collaboration with Dr. Michael Weinstein at Ohio State University a strain of mice that have the Smad2 gene flanked by loxP sites so that the gene can be selectively eliminated in cells

expressing Cre. This model will allow very precise alterations in gene expression by either knocking out or activating genes in precise groups of cells.

Status of Specific Aim 2.

2a: The inhibition of Smad2 has been accomplished using siRNA technology and the reduction in the protein documented. Replication of the experiments are continuing to generate sufficient data for publication – experiments to be completed and published.

2b: The TGF- β Type III receptor has been specifically localized to proliferating neonatal prostate cells and the function of this receptor in development is being characterized. The phosphorylation of Smad2 has been linked to increased levels of cell proliferation at the earliest stages of organogenesis. The results are being analyzed and replicated to be used in publications of the findings - completed.

2c :An epithelial-specific promoter driving Smad2 gene expression has been introduced into a transgenic mouse model to permit gain-of-function studies that could not be satisfactorily achieved with the adenovirus approach. The effects of enhanced expressed are being analyzed and replicated – animals available and being analyzed.

Publication:

X-M Cui, N Shiomi, C Shuler Expression of TGF- β receptors is developmentally regulated and correlated with Smad2 and not Smad 3 expression (to be submitted) Journal of Cell Biology.

Specific Aim 3. To define the biological function of the feedback inhibitory Smad7 and Smad6 proteins during mouse prostate growth and development in organ culture.

The third specific aim is mechanistic and also based on the descriptive foundation findings in SA1. This aim contains three subaims; 3a) organ-specific effect of gene expression; 3b) effects of inhibiting gene expression; 3c) effects of gene over expression. The induction of the intracellular signaling pathway for TGF- β can be inhibited by Smad6/7. The induction of these inhibitory Smads can be linked to regulation of the propagation of the TGF- β signaling and thus Smad6/7 may be thought of as antagonistic to Smad 2/3, which were the focus of Specific Aim 2. Thus the results of this aim analyze the outcomes in cells and tissues when the intracellular pathway activated by TGF- β ligand binding is inhibited.

The initial characterization of the baseline Smad6/7 expression during organogenesis was included in the foundational studies in Specific Aim 1 (3a). Smad6/7 remain outcome markers that are used in both Specific Aim 2 and Specific Aim 3 to establish the basis for interpretation in experimental tissue with modulated gene expression. The studies in 3b are being initiated and consequently the characterizations in 3a will occupy additional effort and the results will have important meaning to the interpretation of the interventions to alter gene expression and are based on other reports of important aspects of organogenesis.^{5,6}

The use of antisense oligonucleotides was originally proposed to inhibit the Smad6 and Smad7 in the cultured tissues (3b). It was found that antisense oligonucleotides have considerable toxicity as a result of the chemistry of these molecules and that that toxicity can confound the results. A new technology, siRNA, has been developed that accomplishes the same outcome as antisense oligonucleotides, to knock-down the level of gene expression. We have chosen to shift to the siRNA technology to knock-down the level of gene expression. It is to our

considerable advantage that the Dharmacon Corporation has developed a full set of siRNA for the TGF- β signaling pathway molecules including Smad2,3,4,6 & 7. Thus we are now using this more effective technology to evaluate the effects of knocking down gene expression. As reported in the October 14, 2004 Progress Report we have optimized the siRNA technology for use in the organ culture model system. "One approach to determine the importance of a molecule in the events required for organogenesis is to selectively eliminate that molecule from the cell and observe the effects. Since the Smad molecules are intracellular there is a requirement to accomplish their removal through methods that interrupt either the generation of specific mRNA or translation of the specific mRNA. In the current period of study we have been optimizing techniques to eliminate the signaling through methods using siRNA (short interfering RNA)." We now will proceed to use this optimized technology to analyze the Smad6 and Smad7 effects in organ culture.

A complication was found in evaluating the use of the adenovirus system in the organ cultures that was related to the timing of gene expression from DNA transferred to cells by the adenovirus (3c). It was found that the infection of the cell by the virus occurred readily however the movement of the new DNA elements to the nucleus and subsequent expression of the new genes took a period of time. In the rapidly developing organs the availability of new protein from the DNA transferred by the adenovirus often was not present at sufficient levels at the correct period of time to be effective. This was the same problem observed working on studies in Specific Aim 2. In Specific Aim 2 we were able to develop a keratin gene promoter driving Smad2 to achieve an alternative means of over-expression to enable to accomplish our aims. Alternative approaches for Smad6 and Smad7 over-expression are being investigated. When those approaches are developed it will be possible to assess the impact of Smad 6/7 overexpression on organogenesis.

Status of Specific Aim 3.

3a: The localization of Smad6/7 in the tissues was accomplished as part of SA1. Further analysis of the role of these inhibitory Smads is coordinated with the other experiments using the SA1 findings as outcome markers for control and experimental tissues. The Smad6/7 patterns will be compared with the other molecules in the TGF- β signaling pathway – localization completed.

3b: The inhibition of Smad6/7 by siRNA is being initiated. The siRNA technology has been established in the laboratory and the reagents are available from Dharmacon company. The impact of inhibition of Smad6/7 will be analyzed during organogenesis – studies on-going and require completion of sufficient replicates for publication.

3c: The adenoviral approach to over-express the genes had a technical problem such that expression did not occur with the appropriate timing. Genetic models for over expression of Smad6/7 are being developed to achieve this sub-aim – alternative models being examined for this sub-aim.

Publication:

The analysis and replication of the results from the studies in Specific Aim 3 has been initiated. The generation of sufficient reproducible data will allow for the generation of a publications.

Key Research Accomplishments:

Temporo-spatial patterns of expression of Smad2 and Smad 3.

Smad 2 is present in the prostate tissues at early stages and is correlated with the presence of the TGF- β receptors on the same cells. Smad 3 has a similar pattern of expression early in the process however it remains expressed in later stages at which time the cells no longer have TGF- β receptors. These results indicate that more than one type of TGF- β superfamily molecule may be involved in epigenetic mechanisms regulating organogenesis.

All three TGF- β cell surface receptors present in developing tissues.

At the P0 stage of development all 3 TGF- β receptors are present in the early organ primordia, indicating that signaling following binding of the ligand can occur through the TGF- β RI and TGF- β RII receptors and that TGF- β RIII has a role that is as yet unknown. Importantly the receptor expression correlates with Smad 2 but not with Smad 3 at the P35 stage suggesting that additional signaling pathways are active at later stages of organ development.

Smad 2 animal model for over expression of the gene

A genetic model for Smad 2 overexpression has been developed based on using an epithelial-specific promoter driving Smad 2 expression. This will allow the effects of Smad 2 over-expression to be examined in the early stages of organ development and forced expression in later stages in which Smad 2 is not identified. This model is currently being actively investigated generating data sets not possible without the specific transgenic animal.

Tissue-specific Selective elimination of Smad 2

Through collaboration with Dr. Michael Weinstein a mouse line has been made available that has the Smad 2 gene surrounded by loxP sites. Breeding this mouse with mice that have the Cre recombinase gene driven by an epithelial-specific promoter will allow selective elimination of Smad 2 and permit that analysis of this effect in vivo. This type of approach provides opportunities based on in vivo genetics that are unique and will provide assessment of the necessity for Smad 2 at early stages in vivo.

Reportable Outcomes:

XM Cui, N Shiomi, CF Shuler Smad 2 and Smad 3 expression critical to prostate organogenesis, (to be submitted) Anatomy and Embryology,

X-M Cui, N Shiomi, C Shuler Expression of TGF- β receptors is developmentally regulated and correlated with Smad2 and not Smad 3 expression (to be submitted) Journal of Cell Biology.

X-M Cui, N Shiomi, C Shuler, Effects of Smad2 overexpression on the development of the prostate gland (in preparation)

X-M Cui, N Shiomi, C Shuler, Tissue-specific deletion of Smad2 at the earliest stages of prostate development arrests organogenesis (in preparation).

Conclusion:

These studies are focused on the specific signal transduction mechanism related to TGF- β binding to specific receptors, subsequent activation of kinase activity and activation of downstream mediators through phosphorylation. The development of the technologies to evaluate loss-of-function and gain-of-function of this signaling pathway will allow focus of specific cell differentiation under the control of TGF- β signaling. The use of novel transgenic mice provides approaches that are focused on specific elements of the signaling pathway. Further investigation of the fundamental mechanisms will provide improved characterization of control of the critical developmental events.

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Appendices:

None